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Biochimica et Biophysica Acta 1783 (2008) 713–727
Biochimica et Biophysica Acta BBA
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# NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK

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Received 5 July 2007; received in revised form 31 December 2007; accepted 3 January 2008
Available online 12 January 2008

**Abstract**

Constitutively activated NF-κB occurs in many inflammatory and tumor tissues. Does it interfere with anti-inflammatory or anti-tumor signaling pathway? Here, we report that NF-κB p65 subunit repressed the Nrf2-antioxidant response element (ARE) pathway at transcriptional level. In the cells where NF-κB and Nrf2 were simultaneously activated, p65 unidirectionally antagonized the transcriptional activity of Nrf2. In the p65-overexpressing cells, the ARE-dependent expression of heme oxygenase-1 was strongly suppressed. However, p65 inhibited the ARE-driven gene transcription in a way that was independent of its own transcriptional activity. Two mechanisms were found to coordinate the p65-mediated repression of ARE: (1) p65 selectively deprives CREB binding protein (CBP) from Nrf2 by competitive interaction with the CH1-KIX domain of CBP, which results in inactivation of Nrf2. The inactivation depends on PKA catalytic subunit-mediated phosphorylation of p65 at S276. (2) p65 promotes recruitment of histone deacetylase 3 (HDAC3), the corepressor, to ARE by facilitating the interaction of HDAC3 with either CBP or MafK, leading to local histone hypoacetylation. This investigation revealed the participation of NF-κB p65 in the negative regulation of Nrf2-ARE signaling, and might provide a new insight into a possible role of NF-κB in suppressing the expression of anti-inflammatory or anti-tumor genes.


*Keywords:* NF-κB; Nrf2; ARE; MafK; CBP; HDAC; Transrepression

## 1. Introduction

NF-κB is a widely expressed, pleiotropic transcription factor. Aberrant NF-κB activity is implicated in diverse pathological processes including infection, inflammation and cancer [1]. NF-κB is composed of homo- and heterodimeric complexes of members of the Rel family of proteins, consisting of p65 (RelA), c-Rel, RelB, p50, and p52. The best-studied and most abundant component of these complexes is the p65/p50 heterodimer. This heterodimer is normally maintained in the cytoplasm by inhibitor molecule IκB. Following a variety of extracellular stimuli such as pro-inflammatory stimuli, genotoxic and oxidative stress, the IκB kinase (IKK) signalosome is activated. Then, the activated IKK phosphorylates and degrades IκB. As a consequence, the p65/p50 complex is liberated, moves to nucleus, binds to the promoter region of the target genes, and induces their expression [2]. In nucleus, p65 concerts gene transcription by recruiting either coactivators, such as CREB binding protein (CBP) [3,4], or corepressors, such as histone deacetylases (HDACs), to target gene [5–8]. It has two opposing effects on gene transcription. Upon activation, p65 drives expression of its target genes, but also suppresses transcription of others. Of note, the transcription mediated by glucocorticoid receptor (GR), PPARγ , MEF2, or p53 has been reported to be repressed by activated p65 [7,9–11]. However, whether NF-κB p65 subunit suppresses the antioxidant responsive element (ARE)-driven gene transcription is unknown.

Nrf2 is a member of the CNC-basic leucine zipper (CNC-bZIP) family of transcription factors, which coordinately regulates the constitutive and inducible expression of antioxidant and phase 2 detoxification enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), via a *cis*-acting DNA element called ARE [12,13]. A bulk of evidence from

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doi:10.1016/j.bbamcr.2008.01.002

transgenic mice has proved that Nrf2-ARE pathway is indispensable for body defense against oxidants, carcinogens, and inflammatory insults [14–16]. Thus, Nrf2-ARE pathway has been proposed to be a promising target for the anti-inflammation and cancer prevention [17,18]. Nrf2 is normally maintained in cytoplasm by its cytosolic repressor protein Keap1 [19,20]. Keap1 negatively regulates Nrf2 by both enhancing its rate of proteasomal degradation and altering its subcellular distribution under oxidative, inflammatory or toxic stress [21]. As a result, Nrf2 translocates to nucleus, forms heterodimer with small Maf protein (MafK, MafG or MafF) and binds to ARE sequences in the promoter region of the genes which encodes antioxidant and detoxification enzymes [22]. Although it was recently reported that some nuclear proteins, such as Bach1 [23] and estrogen receptor [24], antagonize Nrf2 activity, there has been no report on the interference of NF-κB with Nrf2-ARE signaling.

Since a variety of anti-inflammatory or anti-carcinogenetic phytochemicals suppresses NF-κB signaling and activates Nrf2-ARE pathway as well [25–27], one may ask if the suppression of NF-κB signaling and the activation of Nrf2-ARE pathway are totally independent or may crosstalk with each other in some way. On the other hand, as NF-κB and Nrf2 plays contrary role in the pathological processes of inflammation and cancer, and a large number of pathological stimuli, such as cigarette smoke, lipopolysaccharide (LPS), flow shear stresses, oxidized low-density lipoprotein, and reactive oxygen species, activates both NF-κB signaling and Nrf2-ARE pathway [28–35], one may also ask if there is any regulatory mechanism to integrate these two functionally opposing pathways and finally determine the transcriptional outputs? Based on above considerations, we postulate a crosstalk between NF-κB and Nrf2 signaling. In this investigation, NF-κB p65 subunit was found to unidirectionally antagonize ARE-mediated gene transcription through two distinct but interconnected mechanisms.

## 2. Materials and methods

### 2.1. Chemicals, antibodies and plasmids

12-*O*-tetradecanoylphorbol-13-acetate (PMA), *tert*-butylhydroquinone (t-BHQ), suforaphane (SFN), cadmium chloride ($Cd^{2+}$), trichostatin A (TSA), MG132, antibodies against HA and Flag, and other unspecified reagents were obtained from Sigma. Antibodies against Gal4-DBD, HO-1, MafK, c-Myc, CBP, HDAC3, Nrf2, and p65 were purchased from Santa Cruz. Anti-acetylated Lysine antibody was purchased from Cell Signaling Technology. Anti-acetylated histone H4 antibody was purchased from Upstate. Gal4-MafK serial deletion mutants were made by subcloning the corresponding rat MafK cDNA fragment into the pBIND vector (Promega). The following plasmids used in this study were obtained as gifts: NQO1(ARE)-Luc [36], NQO1(ARE-mut)-Luc, HO-1(ARE)-Luc, GPX2(ARE)-Luc [34]; pHO-1(E1)-Luc, pHO-1(E1-ARE mut)-Luc [38], pSilencer3.1-p65 siRNA vector [39], myc-p65, p65(S205A), p65(S276A), p65(S281A)[40], p65(S536A) [41], p65 truncated mutants [42], V5-MafK [43], Gal4-Nrf2(TAD, 1–317aa) [44], Nrf2(E82G) [45], RSV-E1A, HA-CBP [46], GST-CBP(116-738) [47], PKAc(WT), PKAc(K72H) [48], HDAC4/5 [49], pNF-κB-luciferase was obtained from Clontech.

### 2.2. Cell culture and transfection

HepG2, HEK293, and L929 cells were cultured in DMEM medium containing 10% fetal calf serum (FCS) at 37 °C in $CO_2$ incubator. Cell transfection was performed with the FuGENE 6 (Roche) transfection reagent (for HepG2 cells) or JetPei (Polyplus) transfection reagent (for HEK293 and L929cells). The p65-stably expressed L929 cells were obtained by transfection of Myc-p65 vector and selection with 300 μg/ml G418. The transient transfection and luciferase reporter analysis were carried out as previously described [50]. In brief, cells were transfected with various combinations of a luciferase reporter plasmid and desired expression vectors (The amounts of each kind of expression vectors were equal if it is not specified) as well as CMV-β-galactosidase expression plasmid as internal control. Total DNA quantity was kept constant by adding proper amount of pcDNA3 or relevant empty vectors. The luciferase activity normalized against the β-galactosidase activity represents the transcriptional efficiency of the reporter. All transfection throughout this investigation were performed in triplicate, and each assay was repeated at least three times. Results are presented as the folds of induction for the respective vectors or reagents. All data are shown as the mean ± SD of three independent experiments.

### 2.3. RNA interference

The siRNA for p65 and the nonspecific oligonucleotide (mock RNA) are 5′-GAUCAAUGGCUACACAGGA-3′ [39] and 5′-UUCUCCGAACGUGUCACGU-3′ respectively. They were synthesized by GenPharma. HepG2 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen), and all experiments with knockdown of p65 were performed 72 h after transfection. In reporter assay, siRNA expression vector-based p65 knockdown was employed.

### 2.4. Reverse transcription (RT)-PCR

Total RNA isolated from HepG2 cells was reverse-transcribed to cDNA by RT-PCR using the following primers:5′-GAGACGGCTTCAAGCTGGTGATG-3′/5′-GAACTTGGTGGCACTGGCAATG-3′ for HO-1, and 5′-GAAGCATTTGCGGTGGACCA-3′/5′-TCCTGTGGCATCCACCAAAC-3′for β-actin.

### 2.5. GST pull-down, immunoprecipitation, and immunoblotting

The nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) or according to reference [51]. In GST pull-down assay, purified GST-CBP(116–738) or GST protein together with glutathione Sepharose beads (Amersham Pharmacia) was added to 500 μg nuclear extract isolated from transfected HEK293 cells. After gentle rocking at 4 °C for 4 h, Sepharose beads were precipitated by centrifugation and washed 3 times with PBS containing 0.2% NP-40. The proteins were eluted from the beads by adding SDS loading buffer and subjected to immunoblotting analysis with anti-HA or anti-Flag antibodies. In immunoprecipitation assays, corresponding agarose beads-conjugated antibodies were incubated with 500 μg cell lysate in ice-cold lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 1% Triton X-100, 1 mM $Na_3VO_4$, 5% glycerol, and the protease inhibitor cocktail). The lysis buffer used for CBP-HDAC3 interaction is according to reference [52]. The beads were extensively washed with lysis buffer, and the immunoprecipitates were analyzed by immunoblotting with proper antibodies. For immunoblot detection of HO-1 or Nrf2, cells were lysed directly in 2× SDS loading buffer. The whole-cell extracts (WCL) were sonicated and boiled at 100 °C for 20 min prior to loading on SDS-polyacrylamide gels. Procedure for immunoblotting has been described previously [50].

### 2.6. In vivo acetylation/deacetylation analysis

HEK293 cells were transfected with GAL4-MafK alone or cotransfected with GAL4-MafK and CBP and/or HDAC expression vectors. 40 h after transfection, the cells were harvested and lysed in the lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 1% Triton X-100, 1 mM $Na_3VO_4$, 1mM EDTA, 1 mM EGTA, 5% glycerol, and the protease inhibitor cocktail). The lysates were then immunoprecipitated with anti-MafK antibody, and the immunoprecipitates were probed with an anti-pan-Acetyl antibody.

### 2.7. Determination of HDAC activity in vitro

400 μg nuclear extracts of HepG2 cells were isolated and incubated with anti-MafK antibody together with Protein A/G PLUS-Agarose beads (Santa

Cruz) at 4 °C for 4 h. The immunoprecipitates were processed for deacetylation assay using Colorimetric Detection Histone Deacetylase Assay Kit (Upstate Biotech.). The absorbance at 405 nm ($OD_{405}$) of each sample was measured with a Bio-Rad plate Reader and subtracted the background absorbance of Protein A/G PLUS-Agarose beads. Results are presented as fold induction of the net $OD_{450}$ relative to control. All data are shown as the mean±SD of three independent measurements.

### 2.8. DNA affinity pull-down assay

The sense and antisense oligonucleotides for NQO1(ARE) (sense sequence: 5′-CTCAGCCTTCCAAATCGCAGTCACAGTGACTCAGCAGAATC-3′) were synthesized, labeled with biotin at their 5′ end, and then annealed with each other to produce biotin-ARE double-stranded oligonucleotide probe. 500 μg of precleared nuclear extract was incubated with 1 μM biotin-ARE probe and 500 μl streptavidin MagneSphere paramagnetic beads (Promega) in EMSA binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, 2% glycerol and 1 μg poly(dI/dC), pH7.5) at 4 °C for 2 h, and the beads were then extensively washed with the binding buffer. The beads-bound proteins were eluted with SDS loading buffer, and detected by immunoblotting using anti-HA antibody.

### 2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed according to reference with minor modifications [53]. For assay in Fig. 5, $1\times10^7$ HEK293 cells were cotransfected with $(UAS)_5$-TATA-Luc reporter plasmid (3 μg), Gal4-MafK or Gal4 expression vectors (4 μg), together with or without 4 μg p65 expression vector. 40 h after transfection, the ChIP assay was carried out using the ChIP kit (Upstate Biotech) following manufacture-provided protocol. Briefly, cells were treated with 1% formaldehyde to cross-link Gal4 or Gal4 fusion protein to the UAS of pG5-Luc DNA. Then, the cells were harvested, lysed with SDS lysis buffer, and sonicated to shear DNA into fragments between 200 and 1000 bp. The sheared samples were diluted and pre-cleared with Salmon Sperm DNA/Protein A agarose at 4°°C for 30 min. The immunoprecipitation was conducted with anti-Gal4 or anti-CBP antibody, together with Salmon Sperm DNA/Protein A agarose. The immunoprecipitates were eluted from beads using elution buffer, and treated with 20 μl of 5 M NaCl and heated to 65 °C for 4 h to reverse Gal4 or Gal4 fusion polypeptide-DNA cross-link. After treatment with EDTA and proteinase K, DNA was purified. The target sequence in $(UAS)_5$-TATA-Luc was amplified by PCR using a primer set of 5′-GGTACCGAGTTTCTAGACG-3′ and 5′-TTATGTTTTTGGCGTCTTCC-3′. For Fig. 7, the assay was performed in L929 cells with anti-MafK, anti-HDAC3, or anti-acetylated histone H4 antibody using the same protocol. The primer set used to amplify E1 enhancer region of mouse HO-1 promoter are 5′-TGAAGTTAAAGCCGTTCCGG-3′ and 5′-AGCGGCTGGAATGCTGAGT-3′.

## 3. Results

### 3.1. p65 antagonized Nrf2-mediated ARE-dependent gene expression

As a widely used pro-inflammatory and pro-carcinogenetic reagent, PMA can induce nuclear translocation of both p65 and Nrf2 in HepG2 hepatoma cells [54,55]. It was used to stimulate HepG2 cells and see how these two transcriptional factors are activated. The immunoblot analysis showed that PMA stimulation markedly enhanced the nuclear level of p65 and promoted nuclear translocation of Nrf2 within 2 h (Fig. 1A). To evaluate the transcriptional activities of p65 and Nrf2 when both are accumulated in nucleus, HepG2 cells were transfected with NF-κB-Luc or HO-1(ARE)-Luc reporter, and then stimulated by PMA. It was found that PMA effectively induced NF-κB transactivation (15-fold of induction), but only slightly activated ARE-driven gene transcription (1.5-fold) (Fig. 1B). The relative weak activation of Nrf2 may indicate that PMA is not a prototypic activator of Nrf2. To assess the possibility that activated NF-κB may antagonize Nrf2-mediated activation of ARE, the ARE transactivity was examined when NF-κB signaling was enhanced or blocked. At first, the HO-1(ARE)-Luc reporter and the expression vector for either of IKKβ, p65 and p50 were cotransfected into HepG2 cells to see if the cotransfected p65 could affect the PMA-induced expression of the ARE-driven luciferase. As shown in Fig. 1C, the cotransfected p65 or its upstream kinase IKKβ, but not p50, substantially repressed PMA-induced transactivation of ARE, which indicates that the NF-κB p65 subunit suppresses the ARE transactivity. Then, it was examined if ARE transactivity was also changed in the cells having their endogenous p65 knocked down. As Fig. 1D shows, depletion of the endogenous p65 markedly enhanced the PMA-induced expression of the HO-1(ARE)-driven reporter. Meanwhile, the effect of the transfected Nrf2 on NF-κB transactivity was investigated in order to know if Nrf2 also antagonized NF-κB transactivity using pNF-κB-luciferase reporter consisting of four sequential κB consensus sites (GGGAATTTCC) upstream the herpes simplex virus-TK TATA-like promoter. It was turned out that overexpression of Nrf2 did not show any interference with the PMA-induced NF-κB activity (Fig. 1E). These observations clearly demonstrate that p65 unidirectionally antagonizes Nrf2-mediated ARE transactivation if they were simultaneously accumulated in nucleus.

Oxidative and toxic insults usually occur in inflammatory and tumor tissues where p65 is constitutively accumulated in cell nuclei [56–60]. In order to establish a connection between constitutive activation of p65 and the expression of ARE-driven antioxidant gene, the protein level of HO-1 was examined in the p65-overexpressing cells in which p65 is mainly localized in nucleus due to relative deficiency of cytosolic IκB (data not shown). Cadmium ion ($Cd^{2+}$), a pro-inflammatory and pro-carcinogenetic heavy mental ion, was used to induce HO-1 expression [38]. Western blot analysis revealed that the p65 overexpressed either in stable transfected L929 cells or in transient transfected HEK293 cells substantially inhibited $Cd^{2+}$-stimulated HO-1 expression (Fig. 2A). As control, similar suppression of HO-1 induction was observed in the cells over-expressing a dominant-negative mutant of Nrf2, DN-Nrf2 (see lower panel in Fig. 2A). To know whether p65 represses HO-1 expression through interference with Nrf2-ARE pathway, the reporter, pHO-1(E1)-Luc, containing a E1 enhancer that consists of three AREs at 5′-terminal of HO-1 gene and mediates $Cd^{2+}$-induced activation of HO-1 gene [38], was transfected into HEK293 cells to see if increased p65 could inhibit the transactivation of this enhancer. As Fig. 2B shows, cadmium ions caused 4-fold increase of the E1 enhancer-driven HO-1 minimal promoter-mediated transcription of the luciferase. As expected, the transcription was completely abrogated by cotransfection of p65 or DN-Nrf2. To further confirm the repression of ARE-driven gene expression by p65, more efficient Nrf2 activators, hemin [61], *tert*-butylhydroquinone (tBHQ) [62] and sulforaphane (SFN) [63], were used to stimulate the cells in which either pHO-1(E1)-Luc or pNQO1 (ARE)-Luc reporter was cotransfected with p65 or DN-Nrf2. Similar to the stimulation by $Cd^{2+}$, the hemin-induced (Fig. 2C)

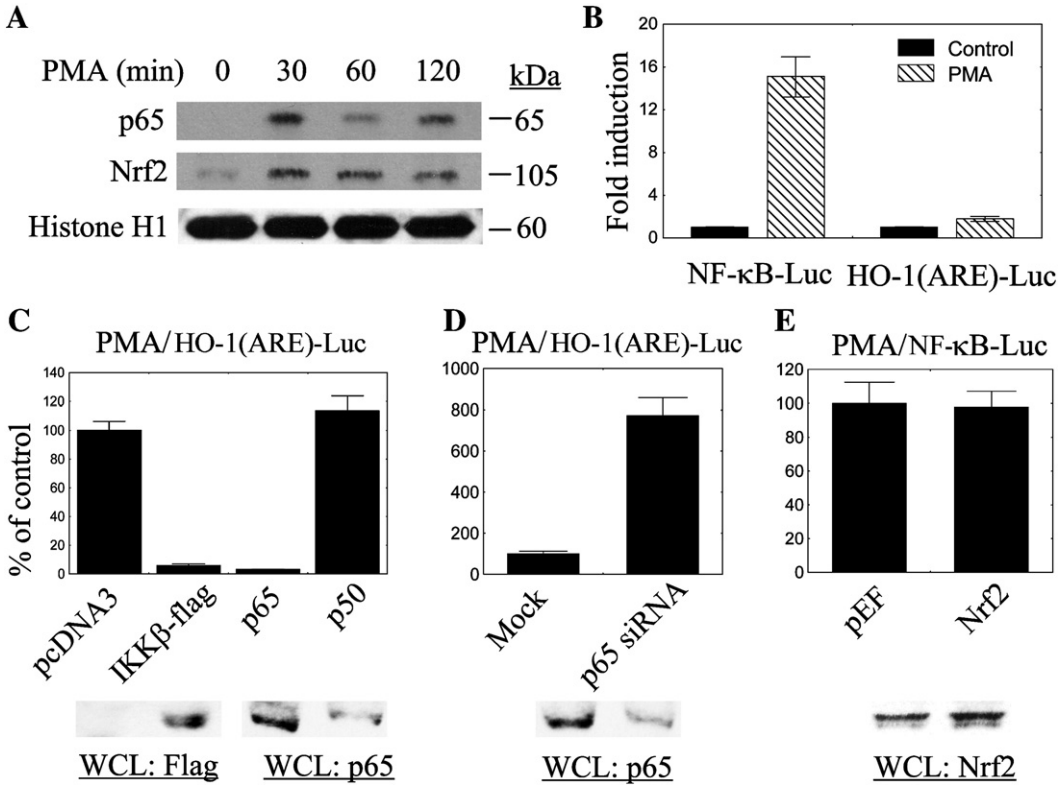


Fig. 1. p65 antagonizes PMA-induced ARE activation. (A) Immunoblot analysis of p65, Nrf2 and histone H1 (control) in the nuclear extracts isolated from the HepG2 cells at indicated time after stimulation with 100 nM PMA. The immunoblot for histone H1 was used to indicate equal loading of the sample in each lane. (B) Differential response of NF-κB and ARE-driven reporter towards PMA stimulation. HepG2 cells transfected with NF-κB-Luc or ARE-Luc reporter plasmid were stimulated with 100 nM PMA for 8 h. The luciferase activities were determined and normalized against the β-galactosidase activity. (C) Effects of IKKβ, p65 and p50 on the PMA-induced transcription of the HO-1(ARE)-Luc reporter in HepG2 cells. (D) PMA induced transcription of the HO-1(ARE)-Luc reporter in the HepG2 cells where the endogenous p65 was knocked down by p65 siRNA. (E) PMA induced transcription of the NF-κB-Luc reporter in the HepG2 cells cotransfected with either Nrf2 or the corresponding empty vector pEF. In the all experiments shown in C–E, the HepG2 cells were cotransfected with HO-1(ARE)-Luc or NF-κB-Luc reporter plasmid together with indicated expression vectors (IKKβ, p65, p50, and Nrf2 for 40 h, and p65 siRNA vector for 72 h) and then stimulated with PMA for 8 h. The cells cotransfected with reporter gene and corresponding empty or mock vector (pcDNA3, pSilencer-mock, pEF) were used as controls. In addition, the whole cell lysate (WCL) (for C, D, E) were isolated under each transfection condition, and subjected to immunoblot analysis for p65 (for C, D), Flag-IKKβ (for C) or Nrf2 (for E) expression. They were shown at bottom of each panel respectively.

and tBHQ- or SFN-induced ARE-driven luciferase expression (Fig. 2D) was completely repressed by cotransfection of p65 or the Nrf2 mutant.

To know if the inhibitory effect of p65 on ARE is dependent on the sequence context of ARE, three luciferase reporters driven by different ARE from HO-1, GPX2, and NQO1 gene or their

Fig. 2. p65 inhibits Nrf2-mediated ARE activation. (A) Effect of overexpressed p65 on the $Cd^{2+}$-induced HO-1 protein expression. The L929 cells stably transfected with myc-p65 and the HEK293 cells transiently transfected with myc-p65 or dominant-negative mutant of Nrf2, Nrf2(DN-Nrf2), were stimulated with $CdCl_2$ (10 μM) for 6 h. The whole cell lysates were then immunoblotted for determining cellular contents of HO-1. (B) Upper panel: Schematic structure of the reporter plasmid pHO-1(E1)-Luc and its mutant, pHO-1(E1-AREs mut)-Luc. Lower panel: Effects of p65 and the Nrf2 dominant-negative mutant on the $Cd^{2+}$-induced transcription of the HO-1(E1) enhancer-driven luciferase gene. HEK293 cells were cotransfected with pHO-1(E1)-Luc reporter plasmid and either p65 or DN-Nrf2 expression vector for 30 h, then stimulated by cadmium chloride (10 μM) for 6 h. The normalized luciferase activities were determined. (C) Effects of p65 and the Nrf2 dominant-negative mutant on the hemin-induced transcription of the HO-1(E1) enhancer-driven luciferase gene. HEK293 cells were cotransfected with pHO-1(E1)-Luc reporter plasmid and either p65 or DN-Nrf2 expression vector for 30 h, then stimulated by hemin (10 μM) for 6 h. The normalized luciferase activities were determined. (D) Effects of p65 and the Nrf2 dominant-negative mutant on the tBHQ or SFN-induced transcription of the NQO1(ARE)-driven luciferase gene. HepG2 cells were cotransfected with NQO1(ARE)-Luc reporter plasmid and either p65 or DN-Nrf2 expression vector for 30 h, then stimulated with tBHQ (100 μM) or SFN(25 μM) for 7 h. The normalized luciferase activities were determined. (E) Repression of the Nrf2-mediated transcription of the HO-1(ARE)-, GPX2(ARE)- and NQO1(ARE)-driven luciferase gene by overexpression of p65. HepG2 cells were cotransfected with indicated reporter plasmid and expression vectors (Nrf2 or Nrf2 plus p65) for 30 h, then normalized luciferase activities were determined. (F) Enhancement of the Nrf2-mediated transcription of the HO-1(ARE)-, GPX2(ARE)- and NQO1(ARE)-driven luciferase gene by knockdown of endogenous p65. HepG2 cells were cotransfected with indicated reporter plasmid and expression vectors for mock RNA, p65 siRNA, Nrf2 plus mock RNA or Nrf2 plus p65 siRNA vector for 72 h. The normalized luciferase activities were then determined. (G) Abrogation of the Nrf2-mediated transcription of HO-1(E1)-driven luciferase gene via mutating the AREs in the reporter. pHO-1(E1)-Luc and its mutant, pHO-1(E1-AREs mut)-Luc, was cotransfected with various combination of Nrf2 and p65 expression vector into HEK293 cells. 30 h after transfection, the normalized luciferase activities were determined. (H) Abrogation of the Nrf2-mediated transcription of NQO1(ARE)-driven luciferase gene via mutating the ARE in the reporter. pNQO1(ARE)-Luc and its mutant pNQO1 (ARE-mut)-Luc was cotransfected with various combination of Nrf2 and p65 expression vector into HapG2 cells. 30 h after transfection, the normalized luciferase activities were determined.

mutants, HO-1(E1-AREs-mt) (the plasmid construction is shown in upper panel of Fig. 2B) and NQO1(ARE-mut), were respectively transfected with Nrf2 and/or p65 expression vector into HepG2 cells. As Fig. 2E shows, all of the three AREs could be effectively activated by Nrf2, and unanimously suppressed by the cotransfected p65. However, overexpression of Nrf2 no longer activated expression of the reporter genes if the AREs or ARE upstream luciferase gene was mutated (Fig. 2G and H). For convenience of comparison, the ARE sequences in the studied reporters were listed in Table 1. Consistently, knockdown of the endogenous p65 by siRNA enhanced the Nrf2-mediated activation of those AREs (Fig. 2F). Similar results were obtained with SH-SY5Y, HeLa, Eahy.926, and A549 cells (data not shown). Taken together, all above results demonstrate that either PMA-stimulated nuclear accumulation of p65 or the ectopic overexpression of p65 effectively blocks the Nrf2-mediated ARE activation.

### *3.2. Repression of ARE by p65 is independent on transcriptional induction of NF-κB target genes*

The effective repression of the ARE-dependent gene expression by p65 may suggest a direct interaction of p65 with some

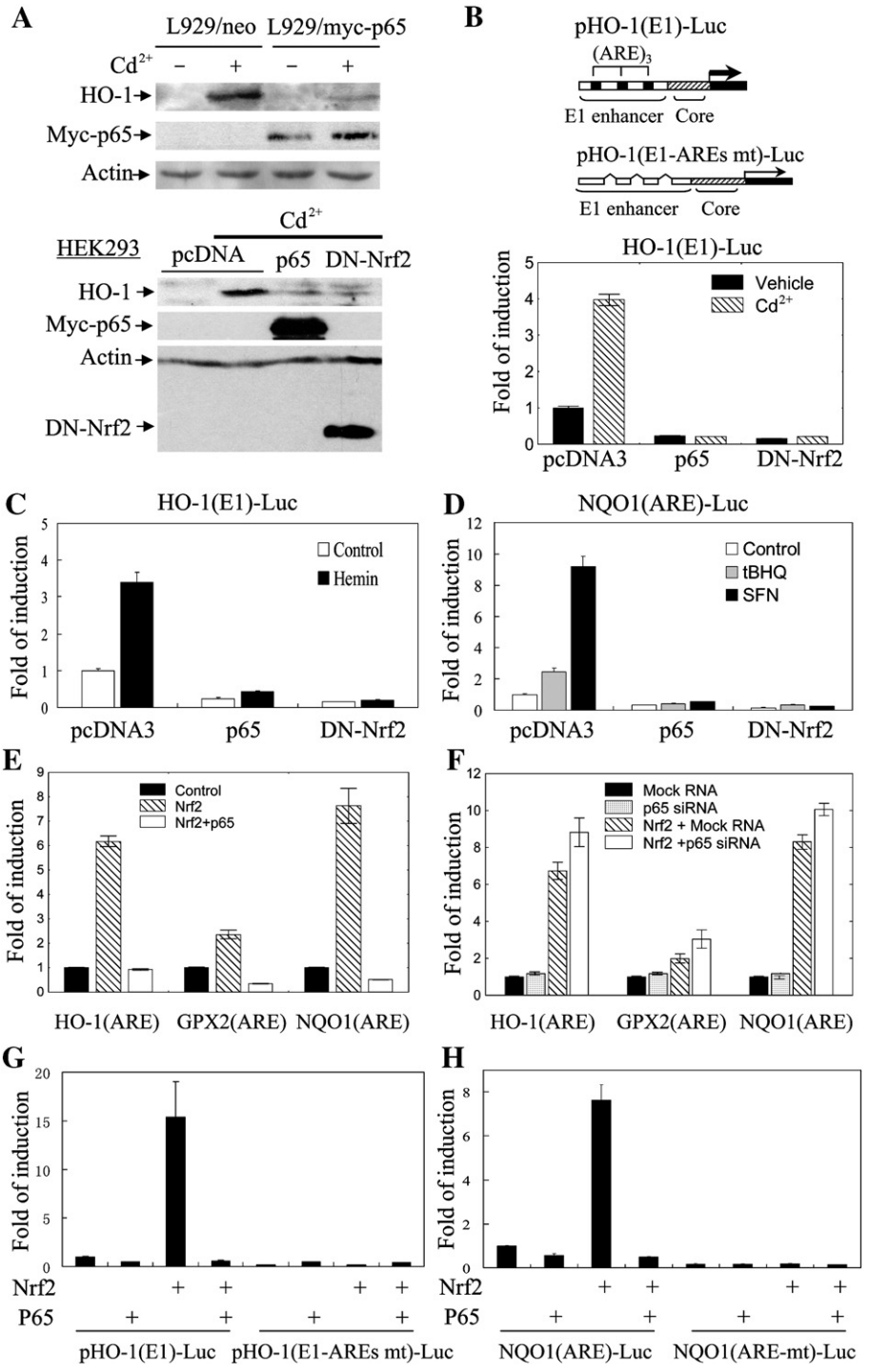

Table 1
Comparison of the ARE sequences in the promoters upstream luciferase gene in various studied reporters with the ARE in mouse NQO1 gene

| Enhancer | Species | ARE sequence in promoter | Reference |
|---|---|---|---|
| HO-1(ARE) | Human | GAACCATGACTCAGCGAAAAC | Banning et al. *Mol. Cell Biol.* 25 (2005) [37] |
| GPX2(ARE) | Human | CCAGGATGACTTAGCAAAAAC | |
| NQO1(ARE) | Rat | TCACAGTGACTCAGCAGAATC | Lee et al. *Biochem. Biophys. Res. Commun.* 280 (2001) [36] |
| NQO1(ARE-mut) | Rat | TCACAGTGACTCAatAGAATC | |
| HO-1(E1) | Mouse | …TGCTGAGTTG…TGCTGTGTCA…TGCTGAGTCA… | Alam et al. *J. Biol. Chem.* 275 (2000) [38] |
| HO-1 (E1-AREs mut) | Mouse | …gctTGAGTTG…TGCgtaGatc…TGCgtAGatc… | |
| Mouse NQO1(ARE) | Mouse | TCACAGTGAGTCGGCAAAATT | Nioi et al. *Biochem. J.* 374 (2003) [13] |

*Note*. The lowercase letters indicate the mutation in ARE. The underlined sequences are the ARE consensus sequences. As the original author indicated, the sequence of mouse NQO1(ARE) and the first ARE sequence of HO-1(E1) are non-classical.

specific proteins involved in Nrf2-ARE pathway. However, co-immunoprecipitation (CO-IP) and fluorescence resonance energy transfer (FRET) studies failed to prove any interaction of p65 with ARE signaling-related proteins such as Keap1, Nrf2, MafK, and Bach1 (data not shown). Besides that, interference of p65 with protein stability, subcellular localization, and DNA-binding activity of Nrf2 was also not observed (data not shown).

p65 contains a C-terminal transactivation domain (TAD) and a N-terminal Rel-homology domain (RHD). To identify the functional domain(s), which is responsible for p65-mediated repression of ARE, a series of C-terminal truncated p65 mutants were transfected respectively with either both HO-1(ARE)-Luc reporter plasmid and Nrf2 expression vector or NF-κB-Luc reporter into HepG2 cells. As Fig. 3A shows, except for the wild-type p65, all of the tested C-terminal TAD-truncated mutants, p65(1–312), p65(1–420) and p65(1–450), lost their capacity to repress ARE (upper panel in Fig. 3A). Western blotting confirmed that all those truncated p65 proteins were expressed at comparable levels as the wild-type p65 protein (Fig. 3E). The results may indicate the necessity of the TAD domain for the transrepression activity of p65.

Since the above TAD-deleted p65 mutants completely lost their ability to transactivate NF-κB target gene (Fig. 3A), could be certain products of the NF-κB-targeted genes responsible for the inhibition of ARE activity? For this reason, three reported transcription-inactive p65 mutants, p65(S205A), p65(S281A) and p65(S536A) [40] was used in the same reporter system to see if they were unable to repress Nrf2-mediated activation of ARE. It was surprisingly found that all of them had similar ability as wild type p65 in repressing the Nrf2-mediated activation of ARE, though they could not activate transcription of the NF-κB regulatory element (see Fig. 3B). At mRNA level, it was found that both p65(WT) and p65(S281A) suppressed Nrf2-induced HO-1 expression to a similar extent (see Fig. 3C). Therefore, these experiments may reveal that p65 itself but not any product of the NF-κB-targeted genes would be responsible for the repression of ARE.

### *3.3. p65 represses Nrf2 transactivity by deprivation of CBP from Nrf2*

As a common coactivator for Nrf2 and p65, CBP is required for the transactivity of Nrf2 [4,44]. Therefore, it was studied if p65 inhibits Nrf2 activity by competitive association with CBP. First of all, the roles of CBP in maintaining p65 and Nrf2 transactivity were compared. Either Nrf2/ARE-Luc or p65/NF-κB-Luc was cotransfected with or without adenovirus E1A, a CBP inhibitory protein [4], into HepG2 cells. Then, the inhibitory effects of E1A on the transcriptional activity of Nrf2 and p65 were determined. As Fig. 4A shows, E1A inhibited transactivity of both p65 and Nrf2 in a dose-dependent manner. However, the Nrf2-mediated transactivation was more sensitive to E1A, suggesting that the transactivity of Nrf2 may be more dependent on CBP.

Since Nrf2 recruits CBP through its N-terminal transactivation domain (TAD) [44], we examined whether p65 could deprive CBP from the TAD of Nrf2. HEK293 cells were transfected with GAL4-Nrf2(TAD) and/or Flag-p65 to see if the cotransfected p65 could affect the association of the endogenous CBP to GAL4-Nrf2(TAD). As the immunoprecipitation analysis in Fig. 4C shows, both Gal4-tagged Nrf2 (TAD) and Flag-tagged p65 were able to associate with the endogenous CBP when each of them was individually expressed. However, once Gal4-Nrf2(TAD) and Falg-p65 were coexpressed, decreased association of CBP with Nrf2(TAD) was observed. It suggests that the increased nuclear p65 may prevent CBP from association with Nrf2(TAD) (lane 2 vs. lane 3 in Fig. 4B). As a positive control, the cotransfected E1A completely abolished the binding of CBP to Nrf2(TAD) (lane 2 vs. lane 4 in Fig. 4B). In a parallel mammalian one-hybrid experiment, it was also found that p65 suppressed the Nrf2(TAD)-mediated transactivation as effectively as E1A.(Fig. 4C).

Domain analysis revealed that the CH1-KIX region on CBP might be a shared region that interacts with either Nrf2 or p65 [41,64,65]. Therefore, it was examined if competition from p65 for this region could lead to dissociation of CBP from Nrf2. GST-pull down assay showed that GST-fused CBP(116-738) fragment, which contains the CH1-KIX region, rather than GST alone specifically interacted with Nrf2 in the nuclear extract of the HA-Nrf2-transfected HEK293 cells (Fig. 4D). However, instead of HA-Nrf2, the Flag-p65 tightly associated with GST-CBP(116–738) when Flag-p65 and HA-Nrf2 were cotransfected in cells. It suggests that p65 deprives CBP from Nrf2 though competition for the CH1-KIX domain of CBP.

Since interaction between p65 and CBP largely depends on the phosphorylation of p65 at S276 that is catalyzed by the catalytic subunit of PKAc [65] it is expected that PKAc should

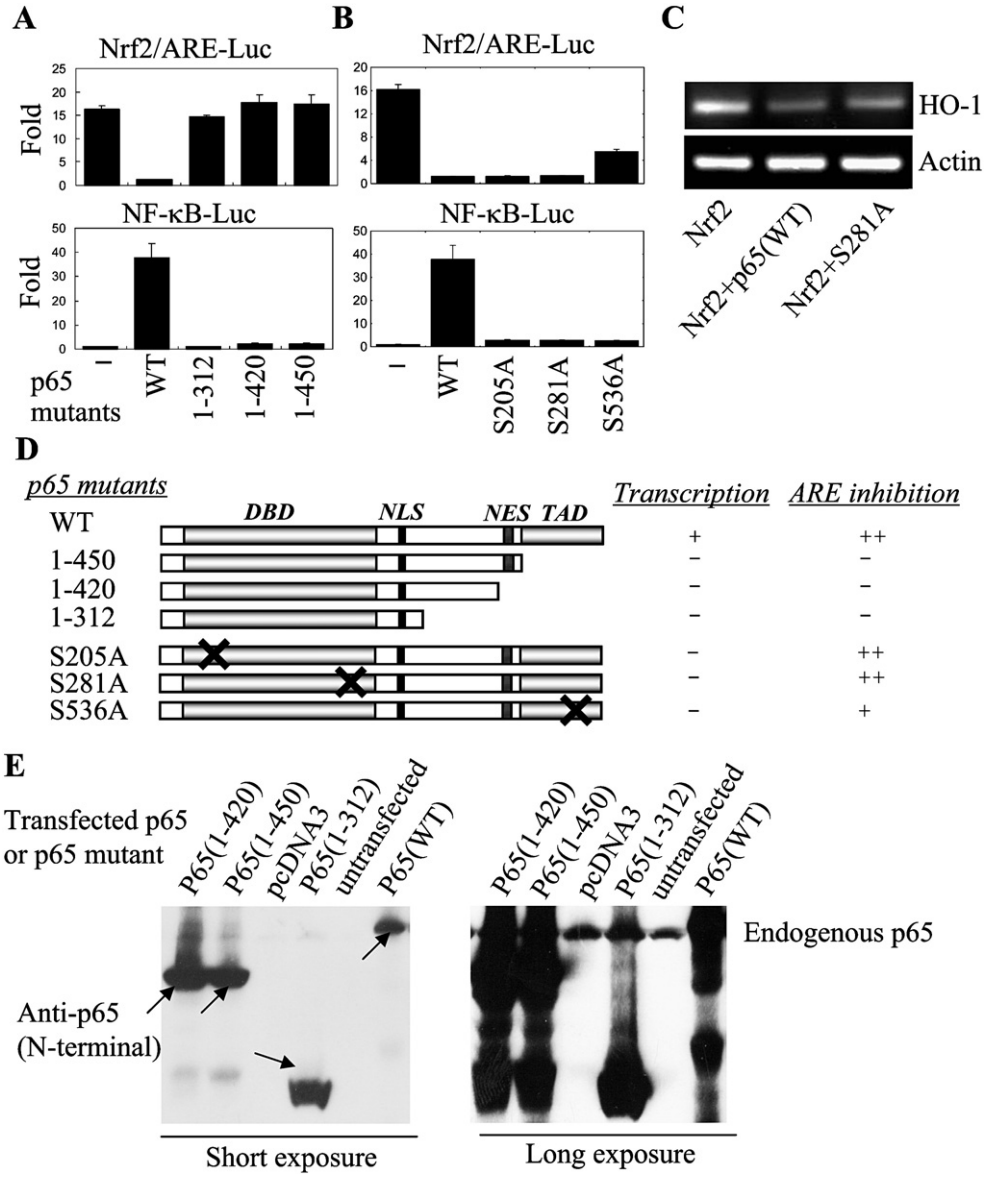


Fig. 3. The effects of p65 mutants on ARE activation. HepG2 cells were cotransfected with ARE-Luc reporter plasmid and Nrf2 expression vector (upper panels in A and B) or NF-κB-Luc reporter plasmid (lower panels in A and B) together with or without the expression vectors of various p65 mutants for 30 h, and then the normalized luciferase activities were determined. Panel (A) and (B) show the effect of the C-terminal truncated, and phosphorylation-deficient p65 mutant on the Nrf2-mediated ARE activation and NF-κB-driven reporter transcription respectively. Panel (C), RT-PCR analysis of HO-1 mRNA level in HepG2 cells transiently transfected with Nrf2, together with or without p65(WT) or p65(S281A). (D) Schematic representation of the p65 mutants constructs used in above experiments. Their abilities in inducing transcription of NF-κB-driven reporter and inhibiting ARE activation are indicated as "+" (significant), "–" (not significant) or "+ +" (very significant). WT represents wild type p65; 1–450, 1–420 and 1–312 represent the C-terminal truncated p65 mutants; S205A, S281A and S536A represent phosphorylation-deficient p65 mutants. (E) Expression of p65 C-terminal truncated mutants in HepG2 cells. HepG2 cells were transfected with indicated expression plasmids. 48 h latter, cells were lysed and subjected to immunoblotting with anti-p65 (N-terminal) antibody. Shorter exposure displays the comparable expression levels of wild-type p65 and p65 mutants. Longer exposure enabled visualization of the endogenous p65.

repress Nrf2 transcriptional activity by enhancing cellular p65-CBP association. Utilizing HO-1(ARE)-Luc as reporter, it was actually observed that overexpression of PKAc significantly inhibited Nrf2-mediated ARE activation, whereas its kinase-dead mutant (PKAc-K72H) had no inhibitory effect (Fig. 4E). It was also found that PKAc even aggravated the inhibitory effect of p65 on the ARE activation (column 3 vs. 5 in Fig. 4E). Nevertheless, the phosphorylation-deficient p65 mutant p65 (S276A) that cannot bind to CBP [65], showed no inhibitory effect on Nrf2(TAD) (Fig. 4F). Consistently, the wild-type p65-mediated inhibition of Nrf2(TAD) was reversed by over-expression of CBP (column 3 vs. 4 in Fig. 4G). These results clearly demonstrate that phosphorylation of p65 at Ser276 is critical for the interaction of p65 with CBP and subsequent p65-mediated repression of Nrf2 transactivation.

Small Maf protein, the DNA-binding partner of Nrf2, has been reported to be essential for Nrf2-mediated activation of ARE [22] and to be able to recruit coactivator CBP [66]. Thus, whether p65 could obstruct MafK-mediated recruitment of CBP to ARE was examined. DNA affinity-pull down experiment was

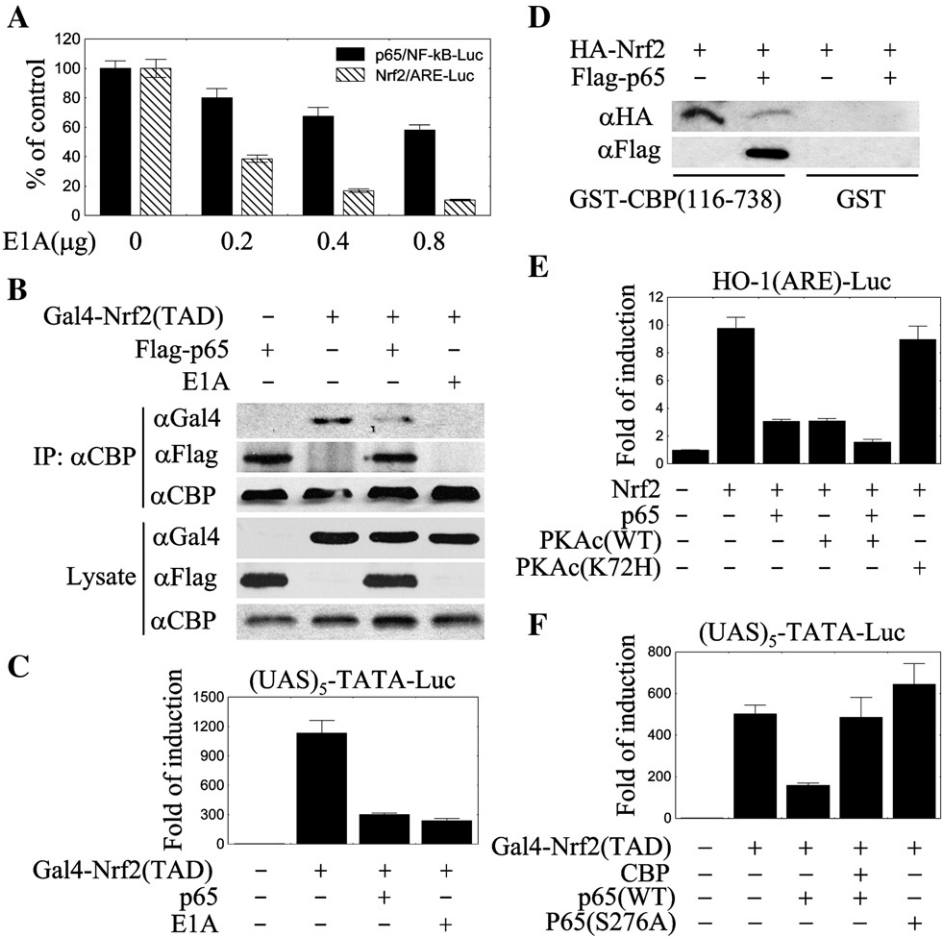


Fig. 4. p65 deprives CBP from Nrf2. (A) Effects of E1A, the CBP inhibitory protein, on p65- and Nrf2-mediated transcription. Either ARE-Luc and Nrf2 or NF-κB-Luc and p65 were cotransfected together with or without indicated amounts of E1A expression vector into HepG2 cells for 30 h, then the normalized luciferase activities were determined. (B) Competition between p65 and Nrf2(TAD) for binding to CBP. HEK293 cells were transiently transfected with indicated plasmids. MG132 (10 μM) was added to the medium during the last 4-h cell culture to prevent proteasomal degradation of transfected Gal4-Nrf2(TAD). Endogenous CBP was immunoprecipitated from the lysates with anti-CBP antibody, and Gal4-Nrf2(TAD), Flag-p65, and CBP in the immunoprecipitates were probed by anti-Gal4, anti-Flag and anti-CBP antibodies, respectively. The expression levels of Gal4-Nrf2(TAD), Flag-p65 and CBP in cell lysates were analyzed by immunoblotting. (C) Inhibitory effect of p65 and E1A on the Nrf2(TAD)-mediated Gal4-based transcription of reporter gene. HepG2 cells were transfected with $(UAS)_5$-TATA-Luc reporter plasmid and indicated expression vectors for 30 h, and then the normalized luciferase activities were determined. (D) Competition of p65 with Nrf2 for binding to CBP(116–738). Nuclear extracts from HEK293 cells transfected with indicated plasmids were incubated with GST-bound or GST-CBP(116–738)-bound glutathione beads at 4 °C for 4 h. The associated Nrf2 and p65 in the precipitates were analyzed by immunoblotting with anti-Flag and anti-HA antibody respectively. (E) Effect of the PKAc on the p65-exerted repression of the Nrf2-mediated transcription of HO-1(ARE)-driven reporter. HepG2 cells were cotransfected with ARE-Luc reporter plasmid and various combination of the expression vectors (800 ng Nrf2, 80 ng p65, and 80 ng PKAc (wild-type or K72H)) for 30 h, and then the normalized luciferase activities were determined. (F) Differential effects of p65 and its phosphorylation-deficient mutant p65(S276A) on the Nrf2(TAD)-mediated Gal4-based transcription of reporter. HepG2 cells were cotransfected with $(UAS)_5$-TATA-Luc reporter plasmid and various combination of the expression vectors (80 ng Gal4-Nrf2(TAD), 80 ng p65 (wild-type or S276A), and 800 ng CBP) for 30 h, and then the normalized luciferase activities were determined.

performed by incubating the biotin-labeled ARE with the nuclear extracts from the HA-CBP-transfected HEK293 cells. As Fig. 5A shows, HA-CBP was obviously attached to the ARE in the presence of both Nrf2 and MafK proteins, and the cotransfected p65 did not interfere with the association of CBP to ARE. Then, a ChIP assay was conducted in the HEK293 cells transfected with $(UAS)_5$-TATA-Luc and Gal4 or Gal4-MafK to see if p65 could interrupt the recruitment of CBP to the DNA-associated MafK. It was found that the endogenous CBP selectively bound to the reporter promoter-associated Gal4-MafK, but not to Gal4 alone. The MafK-CBP interaction was unaffected by the cotransfection of p65 (Fig. 5B). This experiment indicates that p65 is unable to interrupt the association of CBP with MafK, the protein bound to the CH3 domain in CBP [66], despite the fact that p65 effectively deprives CBP from Nrf2.

### 3.4. p65 promotes recruitment of HDAC activity to ARE

Since above-described experiments clearly indicate that the sequestration of CBP is a vital mechanism in the p65-excerted repression of Nrf2, it may be expected that p65(S276A), the CBP-binding-defective p65 mutant [65], would have no effect on the ARE-mediated gene expression. To verify it, the effect of

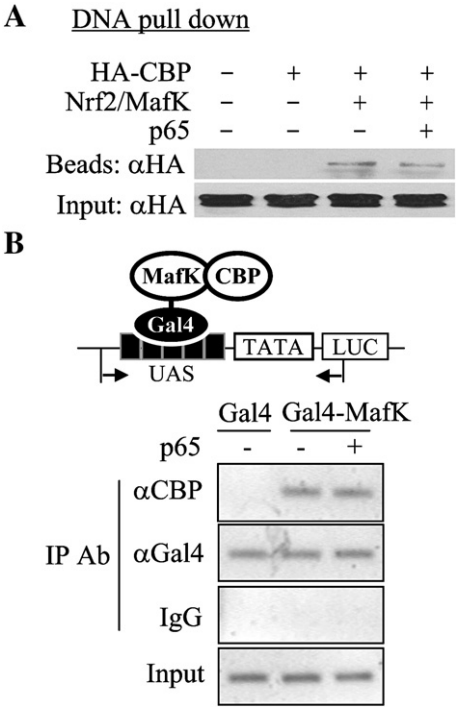


Fig. 5. Association of CBP with MafK and ARE is unaffected by p65. (A) DNA-pull down assay of association of CBP with ARE. After HEK293 cells were transiently transfected with indicated combinations of HA-CBP, Nrf2/MafK and p65 expression vectors for 40 h, the nuclear extracts were incubated with biotin-labeled ARE probe. The CBP proteins coupled to ARE and existing in nuclear extracts were determined respectively by immunoblotting with anti-HA antibody. (B) ChIP assay of the endogenous CBP recruited to Gal4-MafK in the HEK293 cells transfected with $(UAS)_5$-TATA-luc reporter and a combination of p65, Gal4-MafK and Gal4.

p65(S276A) on the basal ARE activity was examined in HepG2 cells. It was surprisingly found that the p65(S276A) still inhibited the ARE-driven transcription, though it was less effective than the wild-type p65 (Fig. 6A). This suggests that there might be any other mechanism involved in the inhibition of ARE by p65. Since it was reported that p65 negatively regulate some transcription factor-mediated gene expression by facilitating the recruitment of the corepressors HDACs [5–8], possible involvement of HDACs in p65-mediated ARE inhibition was investigated. First of all, we examined if trichostatin A (TSA), a HDAC inhibitor, could reverse the effect of p65 on the ARE-mediated gene expression. As shown in Fig. 6A, treatment of the transfected HepG2 cells with TSA moderately reduced the wild type p65-mediated ARE inhibition but completely eliminate the p65(S276A)-mediated ARE inhibition. Therefore, it suggests that the HDACs might be involved in the p65-mediated repression of ARE. Since both Nrf2 and its dimerization partner MafK are ARE-associated proteins, it was then examined if Nrf2 and MafK could serve as adapter to recruit HDACs. For this purpose, HDAC activity was determined in the immunoprecipitate with either anti-Nrf2 or anti-MafK antibody from the nuclear extract of HepG2. As Fig. 6B shows, HDAC activity constitutively associated to MafK rather than to Nrf2. To ascertain which subtype of HDACs could be recruited to MafK, the effect of HDAC1, 2, 3, 4, and 5 on the basal ARE activity was investigated. As shown in Fig. 6C, only class I HDAC (1, 2, and 3) was found obviously inhibit the ARE-dependent gene expression. CO-IP analysis further demonstrated that Gal4-fused MafK, but not naked Gal4, tightly bound to HDAC1 HDAC2, and HDAC3, though a much weaker association of HDAC2 with Gal4 was detected (Fig. 6D). No association of MafK with HDAC4 or HDAC5 was detected (data not shown). As HDACs can deacetylate a bunch of transcription factors and coactivators, it was also examined if HDAC could affect the acetylation status of MafK. Similar to the previous report on MafG by other investigators [60], the overexpression of CBP significantly induced acetylation of MafK in HEK293 cells (see lane 1 vs. lane 2 in Fig. 6E). However, the acetylation could be effectively banned by simultaneous expression of HDAC1 or HDAC3 (see lane 3 and 4 in Fig. 6E).

Due to its highest inhibitory effect on both ARE activation and MafK acetylation (Fig. 6C and E), HDAC3 was chosen to study the regulation of ARE activity by HDACs. First of all, which domain of MafK interacts with HDAC3 was investigated. Various Gal4-tagged MafK fragments were cotransfected with Myc-tagged HDAC3 into HEK293 cells, and the MafK fragment-associated HADC3 was then probed in the immunoprecipitates with anti-Myc antibody from extract of the transfected cells. As shown in Fig. 6F, the C-terminal Zip domain of MafK (77–156 aa), that is known to mediate dimerization with Nrf2, contains the HDAC3 docking site. Therefore, a question was raised. How HDAC3 participates in the p65-mediated repression of ARE? As the ARE-associated MafK and CBP both have a potential to recruit HDAC3 [52], an attempt was made to determine if p65 is capable of promoting them to recruit HDAC3. CO-IP assay was performed in the HEK293 cells cotransfected with various combinations of Gal4-MafK, HA-CBP, Myc-HDAC3, and Myc-p65. As Fig. 7A and B show, both MafK-associated and CBP-associated HDAC3 markedly increased in the presence of the over-expressed p65. It was interestingly found that Myc-p65 and Myc-HDAC3 coexist in the CBP-containing protein complex (Fig. 7B). This observation and the previous reports that p65 can interact with either CBP [4] or with HDAC3 [67], suggest that p65 might facilitate the recruitment of HDAC3 to CBP by serving as a adapter. Meanwhile, the effect of the constitutively activated Nrf2 mutant, Nrf2(E82G), on MafK-HDAC3 and CBP-HDAC3 interactions was examined. The reason for utilizing Nrf2(E82G) but not wild-type Nrf2 is because that the mutant can escape from Keap1-mediated proteasomal degradation in cytosolic retention, thus maximally increases the intra-nuclear Nrf2. As Fig. 7A shows, Nrf2(E82G) completely abrogated HDAC3-MafK interaction possibly due to competition with HDAC3 for binding to the same Zip region of MafK. Similarly, it was also found that the HDAC3-CBP interaction was substantially impaired by Nrf2(E82G) (Fig. 7B). Taken together, these observations suggest that p65 and Nrf2 exert contrary effects on the recruitment of HDAC3 to both MafK and CBP.

To confirm if p65 can promote recruitment of HDAC3 to endogenous ARE under chromosomal environment where the

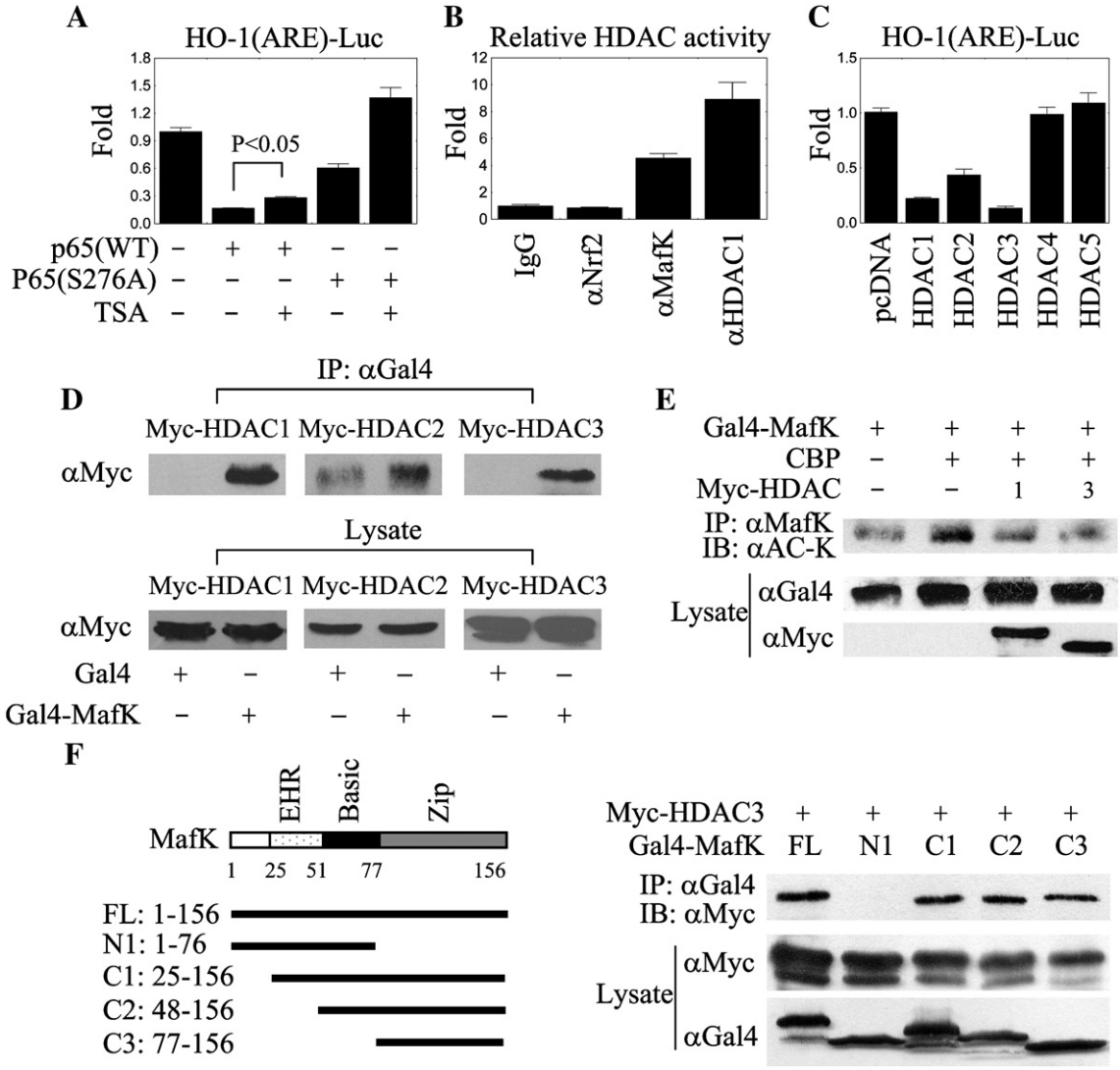


Fig. 6. The MafK mediates recruitment of HDAC to ARE. (A) Effects of TSA on p65- or its mutant p65(S276A)-exerted repression of the HO-1(ARE)-driven transcription of luciferase. HepG2 cells were cotransfected with ARE-Luc reporter plasmid, and the indicated expression vectors for 18 h, followed by treatment with TSA (300 nM) for 12 h, and then the normalized luciferase activities were determined. (B) Determination of Nrf2 or MafK-associated HDAC activity. The nuclear extracts of HepG2 cells were immunoprecipitated with anti-Nrf2 and anti-MafK antibody respectively. The immunoprecipitates were subjected to HDAC activity assay. The immunoprecipitation analysis with IgG and anti-HDAC1 antibody was used as the negative and positive controls respectively. (C) The effects of various subtype of HDACs on basal ARE activity. HepG2 cells were cotransfected with HO-1(ARE)-Luc reporter plasmid, and the indicated expression vector for 30 h, and then the normalized luciferase activities were determined. (D) The MafK-associated HDAC1, 2 and 3 in HEK293 cells transfected transiently with Gal4-MafK or Gal4 expression vector and indicated expression vectors for Myc-HDAC1, HDAC2 and HDAC3 respectively. The HDACs in the immunoprecipitates with anti-Gal4 antibody were probed with anti-Myc antibody (upper blots). The contents of HDAC1, 2 and 3 in cell lysates were determined by immunoblotting respectively (lower blots). (E) Effect of the HDAC1 and 3 on the CBP-mediated MafK acetylation in HEK293 cells. The cells were transfected with various combination of Gal4-MafK, CBP, HDAC1 and HDAC3. The lysates were immunoprecipitated with anti-MafK antibody and then the immunoprecipitates were subjected to immunoblotting with anti-acetylated lysine antibody (αAC-K). The expression levels of MafK, HDAC1 and HDAC3 in cell lysates were immunoblotted with anti-Gal4 and anti-Myc antibody respectively (lower panels). (F) Identification of the MafK domain that may interact with HDAC3. The Gal4-tagged full-length MafK or its domain-truncated mutants, FL, N1, C1, C2 and C3 (see the left panel), were cotransfected with Myc-HDAC3 into HEK293 cells respectively. Associated HDAC3 was probed with anti-Myc antibody in the immunoprecipitates of the lysates with anti-Gal4 antibody from the transfected cells (top blot on the right panel). The expression level of various Gal4-tagged full-length or truncated MafKs and HDAC3 in the transfected cells was also determined (two lower blots on the right panel).

arrangement of *cis*-elements are physiologically normal, the existence of HDAC3 and acetylated H4 histone within the endogenous ARE-enriched HO-1 E1 enhancer was examined by ChIP analysis in the L929 cells transfected with either empty or p65 expression vectors under $Cd^{2+}$-stimulation. As shown in Fig. 7C, a rather low level of HDAC3 was found to associate with endogenous HO-1 E1 enhancer, while the local histone H4 was highly acetylated in control cells (lane 1). In contrast, the HO-1 E1 enhancer-associated HDAC3 was substantially enhanced and the histone H4 was much less acetylated in the p65-overexpressing cells (lane 2). However, the E1 enhancer-associated MafK was kept constant regardless of the presence of the overexpressed p65. Similar results were also obtained in a ChIP experiment with the HEK293 cells transfected transiently

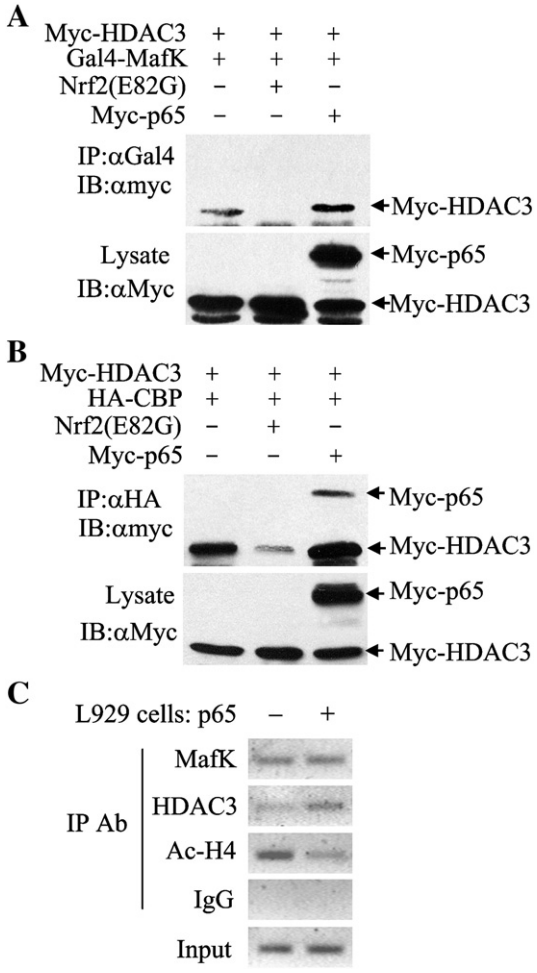


Fig. 7. p65 facilitates association of HDAC3 with either MafK or CBP and promotes recruitment of HDAC3 to ARE. (A) The effects of p65 and Nrf2 on HDAC3-MafK association in the HEK293 cells where Myc-HDAC3 and Gal4-MafK were transfected with or without Myc-p65 or Nrf2(E82G) for 48 h. The MafK-associated HDAC3 was probed by anti-Myc antibody in the immunoprecipitates with anti-Gal4 antibody (the upper blot). The overexpressed HDAC3 and p65 in the transfected cells were probed with anti-Myc antibody (the lower blots). (B) The effects of p65 and Nrf2 on the HDAC3-CBP association in the HEK293 cells where Myc-HDAC3 and HA-CBP were transfected with or without either Myc-p65 or Nrf2(E82G) for 48 h. The CBP-associated HDAC3 was probed by anti-Myc antibody in the immunoprecipitate with anti-HA antibody from the lysates of the transfected cells (the upper blot). The overexpressed HDAC3 and p65 in the transfected cells were probed with anti-Myc antibody (the lower blots). (C) Enhanced association of HDAC3 to HO-1 E1 enhancer by overexpression of p65. The stable Mock- or p65-transfected L929 cells were stimulated with cadmium chloride for 2 h, then the HO-1 E1 enhancer-associated MafK, HDAC3, and acetylated histone H4 were analyzed by ChIP assay using anti-MafK, anti-HDAC3 and anti-acetylated histone 4 antibodies respectively. The ChIP assay with normal IgG was performed to show the specificity of interaction of the indicated proteins with the target region.

with the E1 enhancer-containing plasmid [pHO-1(E1)-Luc] (data not shown). Thus, it was proved that p65 did promote the recruitment of HADC3 to ARE at chromosomal level. The recruitment would reasonably lead to repression of the ARE-dependent gene expression. The ChIP results are consistent with previously observed repression of the $Cd^{2+}$-induced expression of HO-1 in the p65-overexpressing cells (see Fig. 2A).

### *3.5. Knockdown of the endogenous p65 led to more association of CBP to Nrf2 and less recruitment of HDAC to MafK in PMA-stimulated HepG2 cells*

Previous results indicate that both the deprivation of CBP from Nrf2 and the enhanced recruitment of HDAC3 to MafK account for the p65-mediated silence of ARE. To ascertain the existence of such mechanisms in a physiological setting where both NF-κB and Nrf2 pathways are activated, it was investigated how depletion of the endogenous p65 by p65 siRNA affects the CBP-Nrf2 or HDAC3-MafK interaction in the HepG2 cells which were stimulated with PMA. CO-IP was performed to examine if depletion of p65 would modulate level of the endogenous Nrf2-associated CBP and the level of the endogenous MafK-associated HADC3. As Fig. 8B shows, CBP was co-immunoprecipitated with Nrf2, and PMA-stimulation caused more co-immunoprecipitation of CBP (lanes 2–5). 70% depletion of the endogenous p65 markedly increased the Nrf2-associated CBP (lane 2 vs. lane 3), indicating that less p65 results in less deprivation of CBP from Nrf2. The increase caused by depletion of p65 was even more significant under PMA-stimulation, indicating that Nrf2 could capture more CBP upon activation. As to the recruitment of HDAC3 to MafK, it was found that endogenous HADC3 was surely immunoprecipitated with the MafK in the nuclear extracts of HepG2 cells transfected with either p65 siRNA or the mock RNA (Fig. 8C). Although PMA-stimulation slightly enhanced the recruitment of HDAC3 to MafK (lane 3 vs. lane 2 in Fig. 8C), knockdown of the endogenous p65 still led to less association of HADC3 to MafK in the PMA-stimulated cells (lane 4 vs. lane3, Fig. 8C), which indicates that less activation of p65 results in less HADC3 recruited to MafK. In the above CO-IP experiments, normal IgG was used as control, and no detectable CBP or HADC3 was found in the immunoprecipitate with the IgG, indicating that the CBP-Nrf2 and HADC3-MafK interaction are specific. In addition, the MafK-associated HDAC activity was analyzed. As shown in Fig. 8D, knockdown of the endogenous p65 resulted in a clear reduction of MafK-associated HADC activity in either unstimulated or the PMA-stimulated cells. It was noticed that the PMA-stimulation caused significant increase of the MafK-associated HDAC activity in the cells transfection with mock RNA, but the increase was less significant in the p65-knockdown cells These indicate that activated p65 is able to promote recruitment of HDAC3 to MafK, and such a promotion was abrogated when the endogenous p65 was knocked down. Taken together, the opposing results obtained with the p65-knockdown cells are in good agreement with the previous observation that the overexpression of p65 deprived CBP from Nrf2 and promoted the recruitment of HDAC3 to MafK.

## 4. Discussion

Aberrant NF-κB activity is a hallmark of cancer and chronic inflammatory diseases. Constitutive activation of IKK and consequent elevation of the nuclear level of p65 have been reported in many inflammatory diseases and a variety of solid

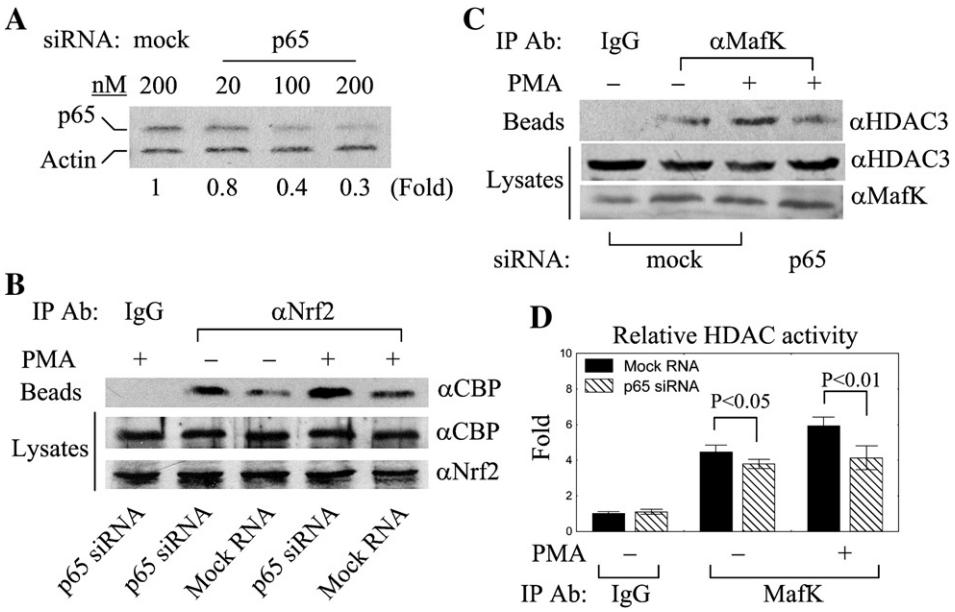


Fig. 8. Effects on PMA-stimulated Nrf2-CBP and MafK-HDAC interactions in HepG2 cells by knockdown of endogenous p65. (A) Expression of p65 in HepG2 cells transfected with various amounts of p65 siRNA for 72 h. Nonspecific double-stranded RNA (Mock) was used as a control, and expression of actin was used as indication of equal loading of samples. (B) The Nrf2-associated CBP in the p65-knockdown cells or control cells stimulated with or without PMA (upper blot). About $6\times10^7$ cells were transfected with 200 nM of p65 siRNA or mock RNA for 72 h, and then stimulated with or without 100 nM PMA for 1 h. The Nrf2-associated CBP was probed by anti-CBP antibody in the immunoprecipitate with anti-Nrf2 antibody or normal IgG in lysates of the cells. Contents of the endogenous CBP and Nrf2 were analyzed by immunoblotting with anti-CBP and anti-Nrf2 antibodies in the lysates (the middle and bottom blots). (C) The MafK-associated HDAC3 in the p65-knockdown cells or control cells stimulated with or without PMA (upper blot). The MafK-associated HDAC3 was probed by anti-HDAC3 antibody in the immunoprecipitate with anti-MafK antibody or normal IgG in the nuclear extracts of the cells transfected with p65 siRNA or mock RNA for 72 h. Contents of the endogenous HDAC3 and MafK were analyzed by immunoblotting with anti-HDAC3 and anti-MafK antibodies in the cell lysates as controls (the middle and bottom blots). (D) Relative activities of the MafK-associated HDAC determined in the immunoprecipitates with anti-MafK antibody or normal IgG in the nuclear extracts of the cells transfected with p65 siRNA or mock RNA for 72 h and then stimulated with or without 100 nM PMA.

tumors [56–58,64]. In this investigation, we revealed that the NF-κB p65 subunit antagonized the anti-inflammatory and anti-carcinogenetic Nrf2-ARE pathway through deprivation of CBP from Nrf2 and promotion of the recruitment of HDAC3 to ARE. As regards the former mechanism, we found that p65 selectively deprived CBP from Nrf2 but not from its DNA-binding partner MafK (Figs. 4 and 5). CBP is a well-established coactivator of Nrf2 and is required for the transcriptional activity of Nrf2 [4,44]. Since this study showed that E1A, the CBP inhibitory protein, almost completely abolished Nrf2-mediated ARE activation (see Fig. S1 in supplementary data), CBP may be considered as the dominant coactivator of Nrf2, and other coactivators of Nrf2, such as recently identified P160/SRC3 [68] and MOZ [69], may function more or less in a CBP-dependent way. Thus, It was suggested that the deprivation of CBP from Nrf2 would substantially impair transcriptional activity of Nrf2. With regard to the latter mechanism, it was found that p65 enhanced the MafK-associated HDAC activity (Figs. 7A, 8C and D) and facilitated the recruitment of endogenous HDAC3 to the inherent AREs-enriched HO-1 E1 enhancer (Fig. 7C). Through this mechanism, recruited HDAC3 deacetylates CBP and abolishes its coactivator activity (see Fig. S2 in supplementary data). The two discovered mechanisms are expected to efficiently repress the ARE-dependent gene transcription. On one hand, the transcriptional activity of Nrf2 is abrogated as a result of CBP sequestration. On the other hand, the recruited HDAC abolish the coactivator activity of CBP by deacetylation [52]. Since optimal co-transcriptional activity of MafG was reported to require the CBP-mediated acetylation [66], the deacetylation of MafK by HDAC3 may be also partly responsible for the repression of ARE. Finally, the recruitment of HDAC activity to ARE may help to maintain local chromosome in a condensed “histone hypoacetylation” state (Fig. 7C), and hence impede local gene transcription [49].

In this investigation, the suppression of the $Cd^{2+}$-induced HO-1 gene expression by overexpression of p65 (see Fig. 2A) was only presented as an example for demonstrating the p65-exerted repression of ARE-dependent gene expression. Since the p65-overexpressing cell system is reminiscent of various inflammatory and tumor-associated tissues, in which p65 is constitutively activated in nucleus, this finding may help us to elucidate why oxidative stresses and toxic insults often occur in those pathological loci. Actually, it would be the most ideal if the repression of the ARE-dependent gene expression by activated p65 could be observed in the presence of physiological agonists of NF-κB. Unfortunately, such an experimental condition seems difficult to meet, because many physiological or pathological stimuli often activate both NF-κB and Nrf2-ARE signaling [28–35]. For example, LPS, an inflammation-related stimulus, activates either Nrf2 or NF-κB in human monocytes [29,31], and flow shear stress, a physiological vascular stimulus, also activates both Nrf2 and NF-κB in human endothelial cells [30,35]. The simultaneously activated Nrf2 may enshroud the inhibitory effect of activated p65 on the ARE-

dependent transcription. Taking PMA, a dual agonist for both NF-κB and Nrf2, as an example, an unchanged expression rather than an "expected" repression of the Nrf2-target genes was observed in PMA-stimulated HepG2 cells (data not shown). Furthermore, the difficulty in demonstrating the repression of the Nrf2 target genes by physiologically activated p65 in a straightforward way may be due to the other fact that some Nrf2 target genes (such as NQO1) also contain potential NF-κB binding site in their promoters [70]. Thus, the repressive effect of p65 on the Nrf2-mediated ARE-driven transcription may be neutralized by the NF-κB-mediated transactivation of the promoter in those genes. The coexistence of different *cis*-elements on a promoter may also explain why siRNA against p65 could not markedly upregulate HO-1 expression (data not shown). Besides that, NF-κB and Nrf2 may cooperate with each other in activating gene transcription in some specific cases. For example, NF-κB and Nrf2 coordinately mediate transcription of Galphai2 gene through simultaneously binding to the adjacent *cis*-elements in the gene promoter [71], and Nrf2 regulates rat glutamate–cysteine ligase catalytic subunit transcription indirectly via NF-κB [72]. Hence, the integrated effect of Nrf2 and p65 on the final output of the ARE-containing gene expression may be stimulus- and/or promoter-dependent. Under physiological conditions, the interference of p65 with Nrf2 transactivation may serve as a negative regulatory mechanism for fine tuning of Nrf2-ARE signaling. If it is true, the antioxidant activity of many natural phytochemicals might be attributed not only to their direct stimulatory effect on Nrf2 signaling but also to their ability in preventing p65-mediated repression of ARE transactivity. However, it should be particularly pointed out that the Nrf2-mediated ARE-driven gene expression would be blocked to some extend by increased nuclear p65 in various inflammatory and cancer tissues where p65 is constitutively activated. Such a blockade may result in a deficiency of cellular antioxidant potential and a subsequent increase of oxidative stress. In turn, the increased oxidative stress may further activate NF-κB [73]. Such a positive feedback mechanism may aggravate the pathological processes of those diseases.

So far, p65 has been reported to repress transcription of some non-NF-κB target genes by either competing for a limited number of nuclear coactivators [11] or serving as a bridge to connect corepressor to target TFs [7]. The present study may show, for the first time, which the two mechanisms are actually coordinated as a concerted process. The p65-mediated ARE inhibition may begin with deprivation of CBP from Nrf2 (TAD) (Fig. 4). This initial step might subsequently result in three potential outcomes. (1) Deprivation of CBP from Nrf2 would lead to a substantial decrease in Nrf2 transitivity (Fig. 4D). (2) Since Nrf2-CBP interaction tends to prevent recruitment of HDAC to CBP (Fig. 7B), disruption of the Nrf2-CBP interaction may restore the ability of CBP to recruit HDAC and therefore link the corepressor to ARE. (3) Deprivation of CBP from Nrf2 but not MafK by p65 may lead to a destabilization of the Nrf2-MafK heterodimer. As a consequence, Nrf2 may lose its inhibitory effect on MafK-HDAC3 interaction (Fig. 7A), and the Zip region of MafK may be partially exposed to HDAC3. Therefore, deprivation of CBP from Nrf2 by p65 may provide a favorable condition for the recruitment of HDAC to both CBP and MafK, thereby resulting in a stable ARE repression. Besides heterodimerization with Nrf2, MafK can also homodimerize at the ARE site [22]. Promotion of HDAC3 recruitment to MafK homodimer by p65 may provide an alternative explanation for p65-exerted ARE repression without involvement of Nrf2.

## Acknowledgements

We particularly thank Drs. M. Yamamoto, J.A. Johnson, R. Brigelius-Flohe, J. Alam, T.D. Gilmore, S. Grant, J. Anrather, C.Y. Sasaki, S.C. Sun, C.B. Pickett, Y. Xiong, MRH White, S. Numazawa, D. Heery, T. Nakajima, Y. Takai, E. Seto, T. Kouzarides, and S.L. Schreiber for their kind help in providing some of the plasmids used in this study. This work was supported by the National Natural Science Foundation of China (No.30330250 and No.30570462) and in part by E-Institutes of Shanghai Municipal Education Commission (project No. E-04010).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.01.002.

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